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Award Number: DAMD17-00-1-0682

TITLE: Beta Human Chorionic Gonadotropin – Induction of Apoptosis in Breast Cancer

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REPORT DATE: January 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
31-01-2006	Final	1 SEP 2000 - 31 DEC 2005

4. TITLE AND SUBTITLE
Beta Human Chorionic Gonadotropin – Induction of Apoptosis in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-00-1-0682

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Kevin J. Cullen, M.D.

Email:

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Maryland
Baltimore, MD 21201

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Agents that induce apoptosis in breast cancer cells have great potential to facilitate chemotherapeutic intervention and improve patient outcomes. In this study, the effects of injecting purified human chorionic gonadotropin (hCG) directly into human breast cancer xenografts grown in nude mice was examined. It was demonstrated that intra-tumoral injection of purified hCG increased the apoptotic index in breast cancer xenografts. These results were supported by the findings that exposure of breast cancer cells to purified hCG decreased cell viability in five different breast cancer cell lines. Further investigation revealed that the expression of Bcl-xL, Bcl-2, and Bax was altered in concert with their role in apoptosis as demonstrated by Western blotting analysis and immunohistochemistry. Preoperative apoptotic induction by factors such as hCG may improve local control, or work synergistically with neoadjuvant chemotherapy to improve complete pathologic response of locally advanced breast cancer.

15. SUBJECT TERMS
Breast cancer, hCG, receptor, Bcl-2, Bax, Bcl-XL, apoptosis, xenografts, gene expression, cell viability

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	59	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

The outcomes of breast cancer treatment dependent greatly on response to therapy and are predicted based on lymph node status and the extent of disease. Locally advanced breast cancers remain difficult to treat, and large tumors are less likely to be resected with negative margins. Although 75-95% of locally advanced breast cancers respond to neoadjuvant chemotherapy, the pathologic complete response (pCR) rate is less than 20%. Since patients with higher pCR rate have improved outcomes, identifying mechanisms to improve the pCR rate may impact on survival of locally advanced breast cancers. Mechanisms that suppress apoptosis are suspected to be significant contributors to the development of intrinsic or acquired resistance to anti-cancer drugs and may prevent complete responses to neoadjuvant chemotherapy. Thus, the induction of apoptosis in breast cancer cells may facilitate therapeutic intervention and potentially improve outcomes. A hormone that could be useful in the induction of apoptosis in breast cancer cells is human chorionic gonadotropin (hCG). In addition to its pregnancy-maintaining actions, hCG causes differentiation of the breast glandular epithelium, which in turn results in 1) inhibition of cell proliferation, 2) increase in DNA repair capabilities of this tissue, and 3) decrease in the binding of carcinogen to the mammary cell DNA (Russo and Russo, 1995; Srivastava *et al.*, 1999). Most hCG actions are mediated by a G-protein-coupled receptor, which also binds luteinizing hormone (LH) (Loosfelt *et al.*, 1989; McFarland *et al.*, 1989). In fact, consistent with hCG function in breast tissue, hCG/LH receptors have been detected in normal breast epithelial cells, breast cancer tissues, and breast cancer cell lines (Hu *et al.*, 1999; Lojun *et al.*, 1997; Meduri *et al.*, 1997, 2003; Span *et al.*, 2003; Taback *et al.*, 2001). This finding is critical considering that it has been proposed that hCG may be useful in the prevention and/or treatment of breast carcinoma (Janssens *et al.*, 2007; Meduri *et al.*, 2003; Rao, 2000; Russo and Russo, 2000). Interestingly, several studies have reported that full-term pregnancy at a young age has a perceived protective effect against the development of breast cancer (Kelsey *et al.*, 1993; Lambe *et al.*, 1996; MacMahon *et al.*, 1970; Medina, 2004; Trapido, 1983). The **purpose** of this study was to determine whether intratumoral injection of purified hCG could increase the apoptotic index in breast cancer xenografts, and to examine whether hCG alters the viability of different breast cancer cell lines. Further, the purpose was to determine if hCG induction of apoptosis was linked to cancer cell expression of estrogen receptor or Her2. The **scope** of the study was to test human breast cancer xenografts for apoptosis and proliferation after direct injection of purified hCG. We chose three cancer subtypes, including Her2 positive cancer (SkBr-3), an ER positive tumor (MCF-7), and an ER/PR negative, Her2 negative cancer (MB-MDA-231). Additionally, we planned to test for cell viability in these cell lines using an MTT assay.

BODY

Task #1: To determine if hCG injection can induce an apoptotic response in breast epithelial carcinoma.

To determine whether hCG could induce apoptosis in breast cancer, we chose to use human cancer cell xenografts grown in nude mice (detailed methods are found in the appended manuscript). Xenografts were created using the human breast cancer cell lines SKBR3, MCF-7, or MB-MDA-231 cells. Initially we planned 3 sets of 5 mice for a total of 15 mice per group (control, vehicle injection, and hCG injection). We modified this plan slightly to provide for internal controls in each animal (bilateral flank xenografts; one treated with vehicle control, and one with hCG) and to increase the n to 10 animals in each of the cell line xenograft. We experienced some difficulties in establishing the xenografts. The MCF-7 group developed metastases, despite the fact that this cell line has not been found to produce metastasis. These animals died before the primary xenografts could be tested. One possible explanation may be "genetic drift" of the cell lines upon multiple passages in culture. The MB-MDA-231 cells did not produce xenografts of sufficient size for testing. The first experiments were performed on SK-Br3 xenografts (Her2 positive cells) only.

Briefly, 50 μL of either 100 IU of semi-purified hCG or saline control was injected into matching flanks of breast cancer xenografts grown in nude mice. After treatment, the tumors were harvested and analyzed by TUNEL assay. The first xenografts (n = 10) were harvested 6 days after injection. The tumors showed substantial necrosis, but no clear evidence of apoptosis, although there were indications of a post-apoptotic effect (some areas appeared to have apoptotic bodies, but did not stain with TUNEL). Additional SkBr-3 xenografts were then established in the remaining 15 animals approved for the protocol. Of these, only 12 xenograft pairs were evaluable. These xenografts were harvested at 24 hrs post injection. It is important to mention that there was evidence of necrosis at the injection site, but clear apoptosis was detected in viable tissue by the TUNEL assay. **Figure 1A** illustrates the results for three representative matched pairs tested using TUNEL assay. As shown, hCG-treated tumors showed 37% apoptosis (range 1-70%) within the xenografts as compared to 14% apoptosis (range 1-20%) observed in saline (control) treated tumor xenographs ($p = 0.001$, **Figure 1B**).

To examine whether the increase in apoptosis seen in **Figure 1** could be matched by an increase in cell proliferation, hCG treated and control xenografts were tested by immunocytochemistry using a Ki-67 specific antibody. Representative images of H&E, TUNEL, and Ki-67 staining are depicted in **Figure 2**. As shown, no differences were observed in cell proliferation as indicated by the Ki-67 staining patterns of saline (control) and hCG treated xenografts. However, a significant increase in TUNEL staining was observed in hCG treated xenografts when compared to control xenografts. Similar results were obtained in the other matched pairs (data not shown). These results suggest that direct, intra-tumoral injection of hCG can induce apoptosis in breast cancer cells without affecting cell proliferation.

To confirm the results obtained with the xenografts, cell viability and proliferation experiments were carried out *in vitro* using different breast cancer cell lines. SKBR3, MCF7, HER18, MDA-MB-231, MDA-MB-435s, MDA-MB-468, and T47D breast cancer cells were cultured in 96 well plates at a density of 1 X 10^4 cells/well and treated with hCG or vehicle control for 6 days as described above. After the incubation period, cells were exposed to MTT dye (5 mg/ml) and incubated at 37^{o}C for 3 hrs. The resulting formazon crystals were solubilized and their absorbance measured at 540 nm as described under Materials and methods. As shown in **Figure 3**, viability decreased in all the cell lines tested. Significant decreases were observed in SKBR3 (22%, $p<0.001$), MDA-MB-231 (18%, $p<0.001$), MDA-MB-435s (14%, $p<0.01$), and T47D (32%, $p<0.001$) (**Figure 3**). This demonstrates that hCG can decrease breast cancer cell viability, and support the induction of apoptosis seen in the xenograft experiments.

To start identifying potential mechanisms involved in the hCG-dependent induction of apoptosis in breast cancer cells, control and treated xenografts were tested for expression of the anti-apoptotic protein Bcl-xL using Western blotting analysis. Only nine pairs of tumor xenografts had sufficient tissue to prepare protein samples. **Figure 4A** depicts the results for matched pairs of xenografts. hCG treatment decreased Bcl-xL protein levels in 6 of the 9 pairs (67%) of xenografts tested, with two pairs without change in expression. In the 9 pairs of xenografts, as shown in **Figure 4B**, hCG treatment decreased Bcl-xL protein levels by 50%. We further tested the xenografts with immunohistochemistry staining (Rubaix, et. Al., 1999) using a Bcl-2 specific antibody demonstrated that this protein was reduced by 53% in the viable tissue around the treatment area, whereas the pro-apoptotic protein Bax was significantly induced 1.5-fold ($P=0.001$) in the treatment area (table 1). To confirm, we also tested SkBr-3 cells in culture. After 6 days of treatment with hCG, a clear reduction in Bcl-XL and induction in Bax protein expression in response to hCG treatment was observed *in vitro* in SKBR3 cells (see **Figure 5**). These results clearly implicate the Bcl-2 family of proteins as a potential pathway for apoptotic induction by hCG.

Task #2: To determine if hCG induction of apoptosis is linked to breast cancer expression of Her2 or ER.

In our proposed plan, we anticipated addressing Task #2 by testing for apoptosis in hCG treated xenografts from human breast cancers that expressed either Her2 (SkBr-3 cells) ER (MCF-7 cells) or neither (MB-MDA-231 cells). As described above, the xenograft model was insufficient to answer this task. We then attempted to determine if hCG could affect cell viability in subtypes of breast cancer, including ER+ and Her2+ cell lines, since our xenograft model was insufficient to determine this biologic effect. As shown in Figure 3, there was no pattern of response clearly patterned by expression of ER or Her2. Her2+ cells responded (SKBr-3) as well as ER+ cells (T47D) and "triple negative" cells (ER-, PR-, Her2-; MDA-MB-231 cells). Studies were then carried out to determine whether the differences in hCG response observed in **Figure 3** were associated with hCG/LH receptor expression in these cell lines. As shown in **Figure 6**, hCG/LH receptors were detected in MDA-MB-231 > SKBR3 > T47D, MDA-MB-468 > MCF7 > HER18. The hCG/LH receptor levels found in the MDA-MB-231 and SKBR3 cells

corresponded with the significant response of these cell lines to hCG treatment. Interestingly, T47D showed the highest response in the MTT studies but had about 50% the amount of hCG/LH receptors found in the SKBR3 cells. Another inconsistency was detected in the case of the MDA-MB-435s cells. These cells demonstrated significant response to hCG treatment in the MTT studies even with undetectable levels of the hCG/LH receptor. Even though MDA-MB-468, MCF7 and HER18 had detectable levels of hCG/LH receptor, their response in the MTT studies was relatively modest. Western blotting analysis was also carried out to confirm the markers expressed in each breast cancer cell line. As shown in **Figure 6**, human epidermal growth factor receptor 2 (Her2) was expressed in SKBR3, HER18, and T47D, whereas estrogen receptor α (ERα) was expressed in MCF7, HER18 and T47D. T47D also expressed both isoforms of the progesterone receptor (PR) (**Figure 6**). None of these protein markers were detected in MDA-MB-231, MDA-MB-435s, and MDA-MB-468 (**Figure 6**). As expected, no ERβ was detected in any of the cell lines under analysis (**Figure 6**).

Future work

Although the proposed xenograft of ER+ and ER-/Her2- cancers were not evaluable for this study, the in vitro work suggests that the apoptotic response to hCG is not dependent on classic subtype expression of ER and Her2. Further characterization of the hCG/LH receptor and possible regulation of its expression by ER or Her2 signaling may provide additional mechanistic insight into responsive tumors. Further, human breast cancer tissues should be tested for expression of hCG/LH receptor and correlative studies with Her2 and ER performed. A pilot study for “proof-of –principle” that hCG can induce apoptosis in human breast cancer is warranted based on these pre-clinical studies.

KEY RESEARCH ACCOMPLISHMENTS

- Treatment of breast cancer with hCG induces apoptosis
- Treatment with hCG does not alter breast cancer cell proliferation (as measured by Ki-67)
- hCG treatment decreases cell viability in breast cancer cell lines
- hCG/LH receptor expression on breast cancer cells corresponded with significant decreases in cell viability
- No correlation of hCG response was seen with Her2 or ER expression
- Bcl-xL expression is decreased, while BAX expression is increased in breast cancer cells after treatment with hCG

REPORTABLE OUTCOMES:

1. Human Chorionic Gonadotropin (hCG) induction of apoptosis in breast cancer. W. Bradford Carter, Madhavi Sekharam, Domenico Coppola, Niranjan Yanamadra. Abstract Presentation at 29th Annual San Antonio Breast Cancer Symposium, December 14-17, 2006. San Antonio, TX (Copy of poster appended to this report)

2. Treatment with Human Chorionic Gonadotropin Induces Apoptosis in Breast Cancer. W. Bradford Carter, Madhavi Sekharam, Domenico Coppola, Dayami Lopez. Oral abstract presentation at 2nd Annual Breast Cancer Research Summit, November 30, 2007. Moffitt Cancer Center, Tampa, FL.

3. Treatment with Human Chorionic Gonadotropin Induces Apoptosis in Breast Cancer. Dayami Lopez, Madhavi Sekharam, Domenico Coppola, W. Bradford Carter. Manuscript submitted for publication review, Molecular and Cellular Endocrinology, December 7, 2007. (Manuscript and figures appended).

CONCLUSIONS

In this report, we have demonstrated that direct injection of hCG into breast carcinoma xenografts induces apoptosis, and that exposure of breast cancer cells to purified hCG decreases cell viability in different breast cancer cell lines. These data are in correlation with previous *in vivo* experiments indicating that hCG can inhibit the progression of 7,12-dimethylbenz anthracene (DMBA) induced mammary carcinomas in rats through induction of apoptosis (Srivastava *et al.*, 1997).

In agreement with our cell viability studies is the report indicating that the culturing of MCF7 cells with hCG results in a hCG/LH receptor-dependent decrease in cell proliferation and invasion across Matrigel membranes (Rao *et al.*, 2004). This finding is interesting considering that it has been shown that women with hCG/LH receptor-positive tumors have longer metastasis-free survival (Meduri *et al.*, 2003). Further confirmation of the effects of hCG in breast cancer was presented in pilot clinical studies demonstrating that hCG significantly reduced the proliferative index and the expression of both ER and PR in breast cancers independently of whether they were newly diagnosed or metastatic (Janssens *et al.*, 2007).

Another finding of the current report is that the response to hCG of several of the breast cancer cell lines tested does not appear to correlate with the expression levels of hCG/LH receptors. One possible explanation is that the endogenous production of hCG in these cell lines masks the effects of the exogenously added hCG. In connection with this possibility, it has been reported that breast cancer cells are able to produce hCG (Bièche *et al.*, 1998). Interestingly, hCG-α is synthesized in high concentrations, especially in ERα-positive tumors (Bièche *et al.*, 1998), which could be associated with the low response of the MCF7 and HER18 cells to hCG treatment shown herein. The finding that T47D, which also express ERα, significantly responded to hCG treatment, could be related to the presence of PR. PR has been shown to regulate the expression of hCG-β (Reimer *et al.*, 2000). One inconsistency was observed in the case of the MDA-MB-435s cells, which had a significant response to hCG treatment in the MTT studies but not hCG/LH receptors. This suggests that hCG may be able to activate a signaling pathway that is independent of the hCG/LH receptor. Further studies are required to examine this possibility.

We also initiated mechanistic studies and determined that Bcl-xL expression was decreased in six of 9 pairs of xenografts as determined by Western blotting analysis. Furthermore, we demonstrated using immunohistochemistry that Bcl-2 was reduced by 53% in the viable tissue around the treatment area, whereas Bax was significantly induced 1.5-fold in the treatment area. Induction in Bcl-XL and reduction in Bax protein expression was also observed in SKBR3 cells treated with hCG. In correlation with our findings, enhancement of Bax protein expression has been detected in hCGβ-expressing breast cancer cells undergoing significant apoptosis (Shi *et al.*, 2006). Studies have shown that Bax acts downstream of the p53-mediated apoptotic pathway (Choudhuri *et al.*, 2002; Medina and Kittrell, 2003; Modestou *et al.*, 2001; Pati *et al.*, 2004; Sivaraman *et al.*, 2001). Interestingly, the absence of p53 function is a known risk factor for

spontaneous tumorigenesis in the mammary gland, and hormonal stimulation enhances tumor risk in p53-null mammary epithelial cells (Medina and Kittrell, 2003; Pati *et al.*, 2004; Sivaraman *et al.*, 2001). In addition to altering the expression of apoptosis related genes, hCG-induced apoptosis appears to involve disruption of N-cadherin-mediated cell-cell adhesion via β-catenin (Pon *et al.*, 2005), activation of the hCG-sensitive cyclooxygenase-2 (COX-2) and gonadotropin-mediated phosphotidyl-inositol-3 kinase pathway (Pon and Wong, 2006), and the induction of the Fas-ligand system (Kayisli *et al.*, 2003). Additional mechanistic studies to define the role of the Bcl-2 family of proteins in hCG-induced apoptosis of breast cancer cells will be needed.

Another tumor that has been reported to be influenced by hCG treatment is the neoplastic Kaposi's sarcoma (KS), the most common tumor found in patients with acquired immune deficiency syndrome (AIDS) (Gill *et al.*, 1996, 1997; Lunardi-Iskandar *et al.*, 1995). Purified hCG has been shown to increase apoptosis in Kaposi's sarcoma cells, both *in vitro* and *in vivo* (Gill *et al.*, 1996, 1997; Lunardi-Iskandar *et al.*, 1995). Interestingly, when highly purified or recombinant hCG and the hCG subunits were used in the studies with KS, no effect was seen (Kachra *et al.*, 1997; Lunardi-Iskandar *et al.*, 1998; Pati *et al.*, 2000; Samaniego *et al.*, 1999). Furthermore, different sources of clinical-grade hCG preparations varied in their anti-KS activity (Pati *et al.*, 2000; Russo *et al.*, 1990; Samaniego *et al.*, 1999; Srivastava *et al.*, 1998a, 1998b, 1999). Attempts to decipher this contradiction lead to the identification of a hCG Associated Factor (HAF) which appears to be responsible for the apoptotic activity of the hCG preparations (Lunardi-Iskandar *et al.*, 1998; Pati *et al.*, 2000; Samaniego *et al.*, 1999). HAF is present in several commercial preparations of hCG, with A.P.L. (Wyeth), the inducing agent in this study, having the most activity. This hCG associated factor could be a peptide, an associated protein, or even a breakdown product of hCG that could be found in the urine of pregnant women (Pati *et al.*, 2000). In fact, it is known that the β subunit of hCG is susceptible to proteolytic cleavage *in vivo* that can produce peptides of the size of the HAF (Lang *et al.*, 1997). Other factors that could be found in commercial hCG in different proportions and have been shown to be toxic to KS cells, include lysosyme, low-molecular weight contaminants, and the eosinophil derived neurotoxin ribonuclease (EDNR) (Kachra *et al.*, 1997; Lang *et al.*, 1997; Masood *et al.*, 1999; Samaniego *et al.*, 1999). Although hCG itself appears to have a direct effect in breast cancer (Rao *et al.*, 2004; Janssens *et al.*, 2007), additional studies are required to identify/purify HAF and to determine its effects in breast cancer cells, either alone or in conjunction with hCG.

In summary, we have identified a significant apoptotic induction in breast cancer xenografts after direct injection of a HAF-containing preparation of hCG. While further characterization of the inducing agents is necessary, these experiments suggest a potential therapeutic advantage by intralesional injection to induce apoptosis in locally advanced breast cancer. Treatment of breast cancer with hCG may increase apoptosis, potentially increasing the pathologic complete response rate of neoadjuvant chemotherapy, and improving prognosis in locally advanced breast cancer.

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SUPPORTING DATA

IHC determination of BCL-2 family proteins in SKBr-3 xenografts

	BAX (% Staining)	Bcl-2 (% Staining)	Bcl-XL (% Staining)
SKBr3 C6	50	50	30
SKBr3 C5	40	50	20
SKBr3 C4	30	30	20
SKBr3 C3	50	60	30
SKBr3 C2	30	40	20
SKBr3 C1	30	3	20
HCG6 SKBr3	30	15	20
HCG5 SKBr3	20	5	5
HCG4 SKBr3	30	20	20
HCG3 SKBr3	20	20	60
HCG2 SKBr3	40	30	60
HCG1 SKBr3	10	5	5
T10 SKBr3 Ct1	40	30	30
T9 SKBr3 Ct1	50	30	50
T7 SKBr3 Ct1	30	30	10
T6 SKBr3 Ct1	70	10	40
T4 SKBr3 Ct1	15	10	5
T3 SKBr3 Ct1	10	10	5
T2 SkBr3 Ct1	5	5	3
HCG8 SKBr3 Dt	20	10	20
HCG7 SKBr3 Dt	10	10	5
HCG6 SKBr3 Dt	40	10	15
HCG4 SKBr3 Dt	5	20	20
HCG3 SKBr3 Dt	5	3	3
HCG2 SKBr3 Dt	10	3	3

TABLE 1

Figure 1

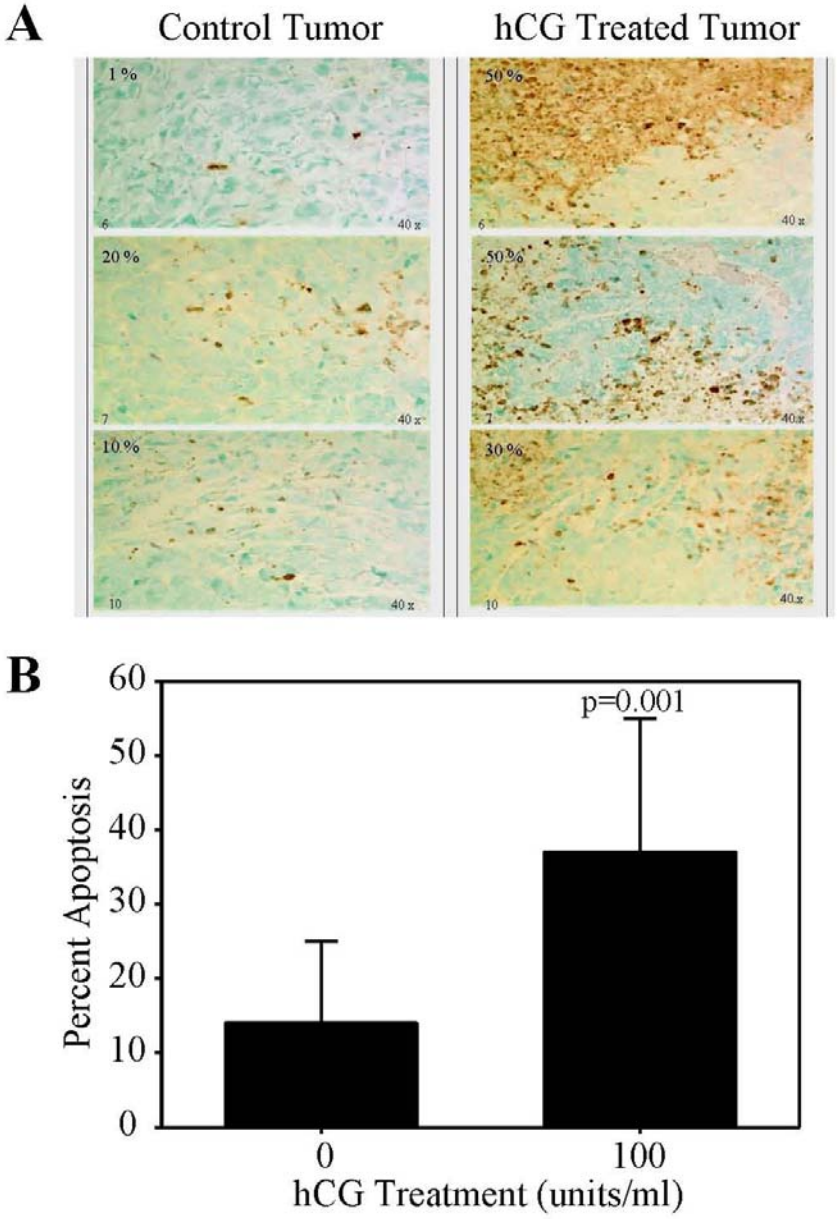

A
Control Tumor
hCG Treated Tumor
1 %
20 %
10 %
50 %
50 %
30 %
B
Percent Apoptosis
60
50
40
30
20
10
0
p=0.001
0
100
hCG Treatment (units/ml)

Figure 2

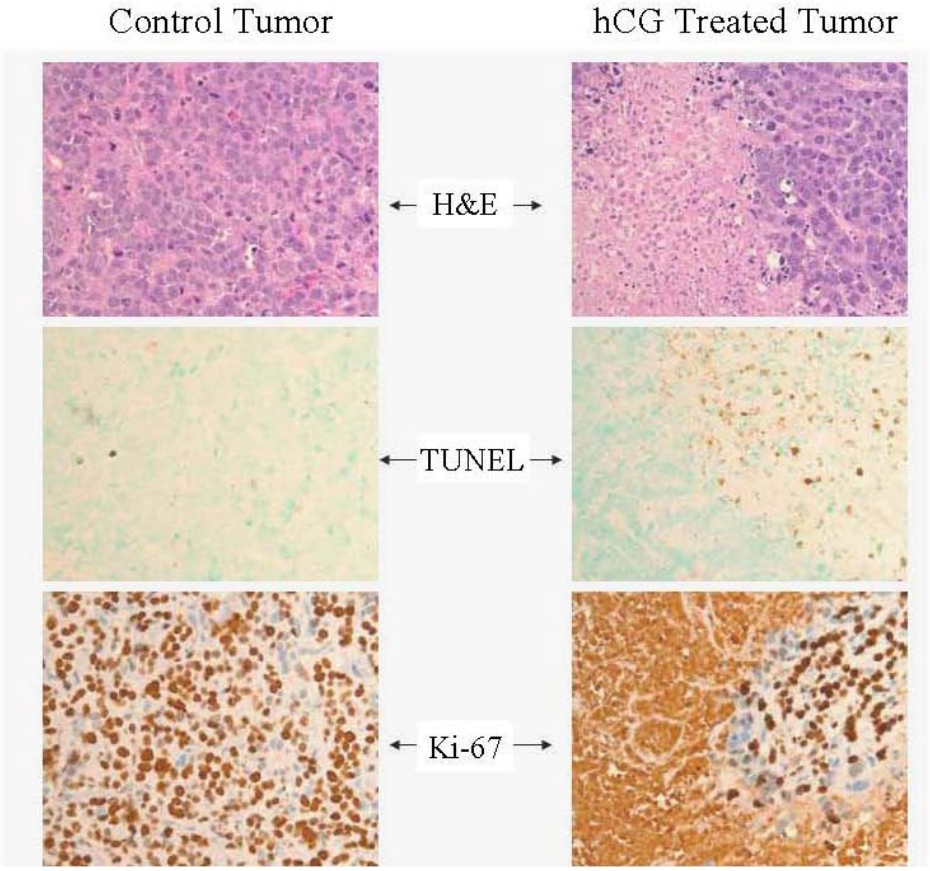


Figure 3

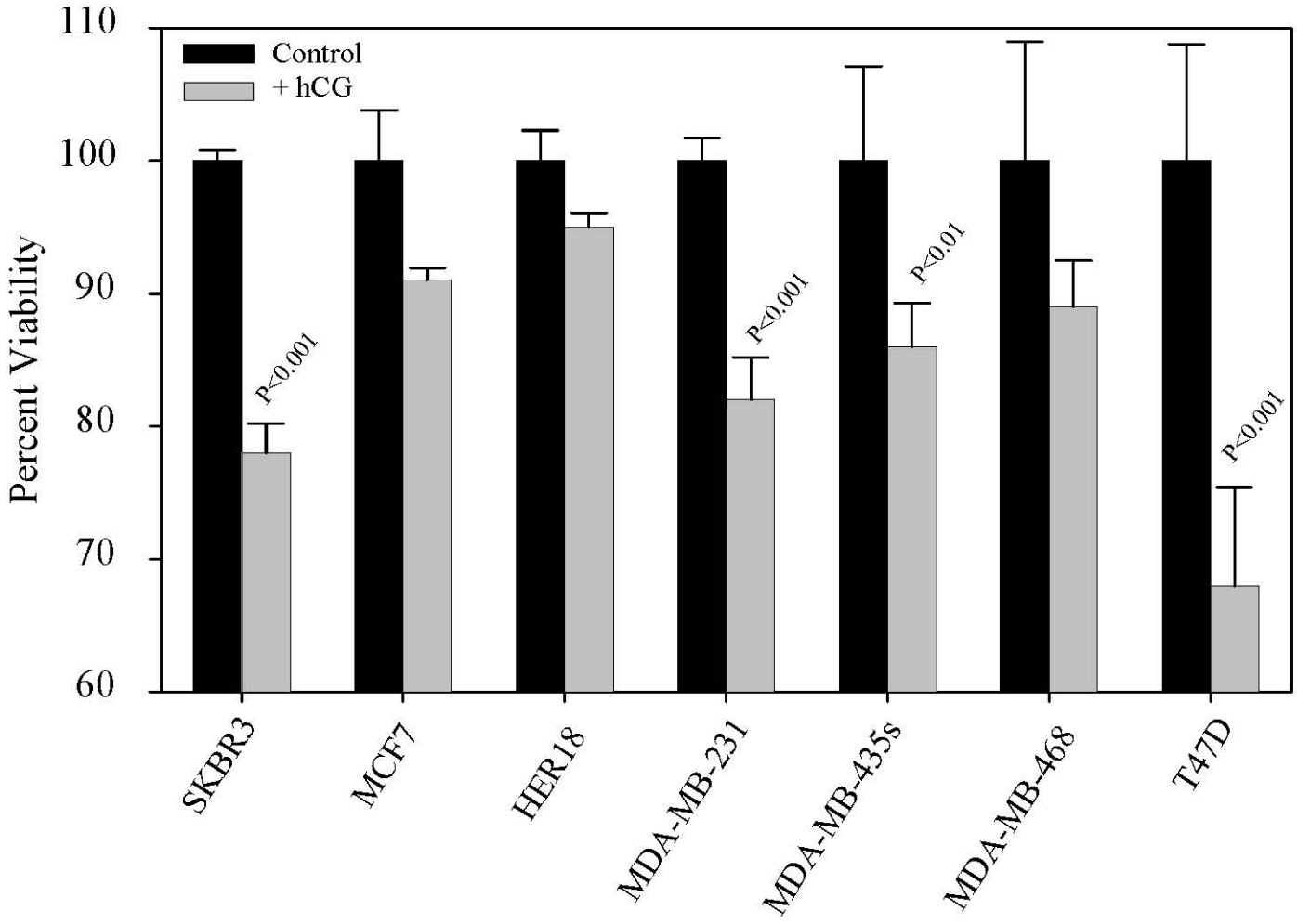

110
100
90
80
70
60
Percent Viability
Control
+ hCG
P<0.001
P<0.001
P<0.01
P<0.001
SKBR3
MCF7
HER18
MDA-MB-231
MDA-MB-435s
MDA-MB-468
T47D

Figure 4A

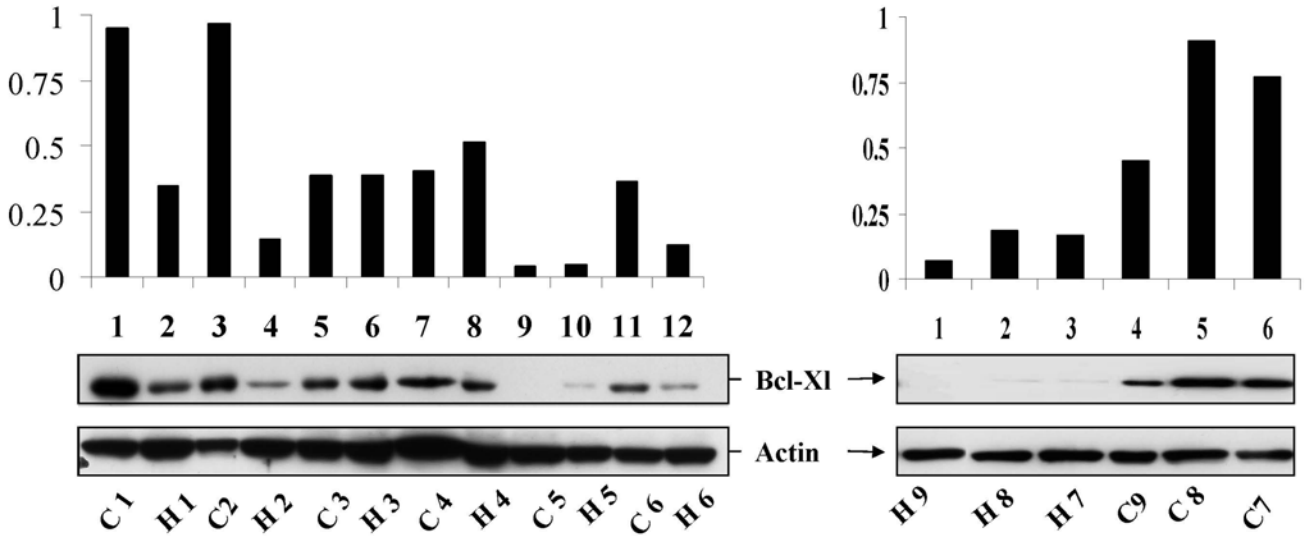

1
0.75
0.5
0.25
0
1 2 3 4 5 6 7 8 9 10 11 12
1
0.75
0.5
0.25
0
1 2 3 4 5 6
Bcl-Xl
Actin
C1 H1 C2 H2 C3 H3 C4 H4 C5 H5 C6 H6
H9 H8 H7 C9 C8 C7

Figure 4B

B

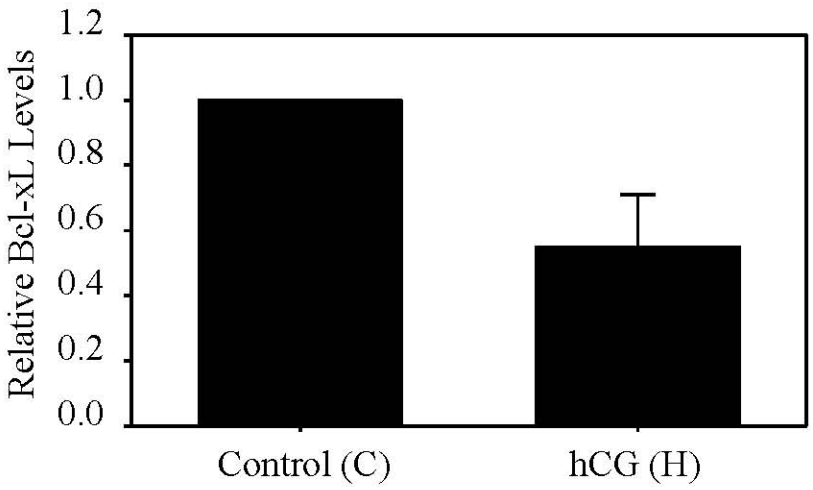


Figure 5

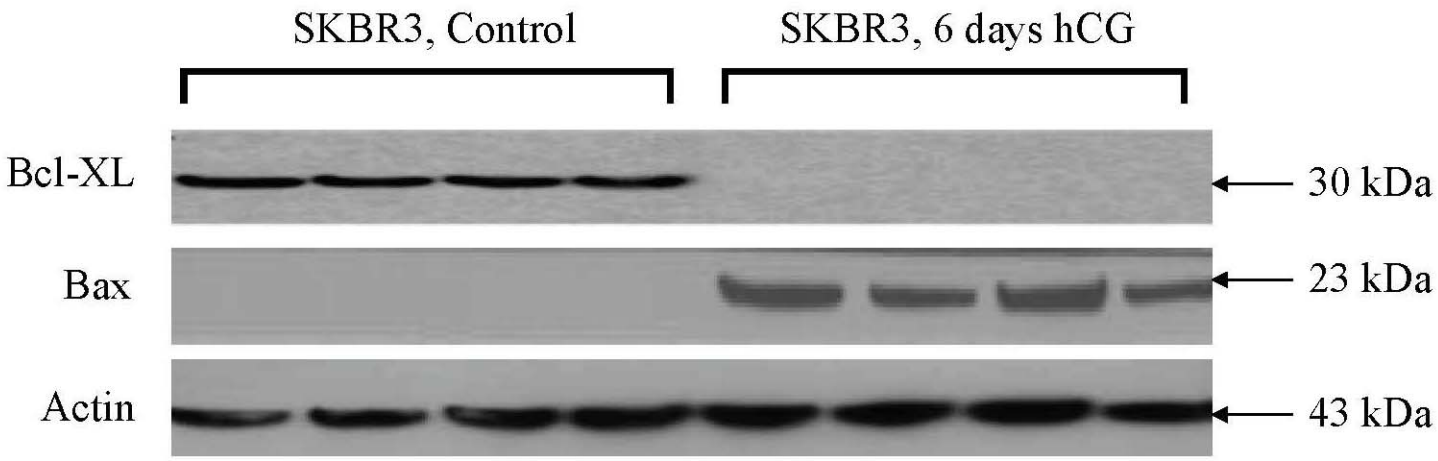

SKBR3, Control
SKBR3, 6 days hCG
Bcl-XL
Bax
Actin
30 kDa
23 kDa
43 kDa

Figure 6

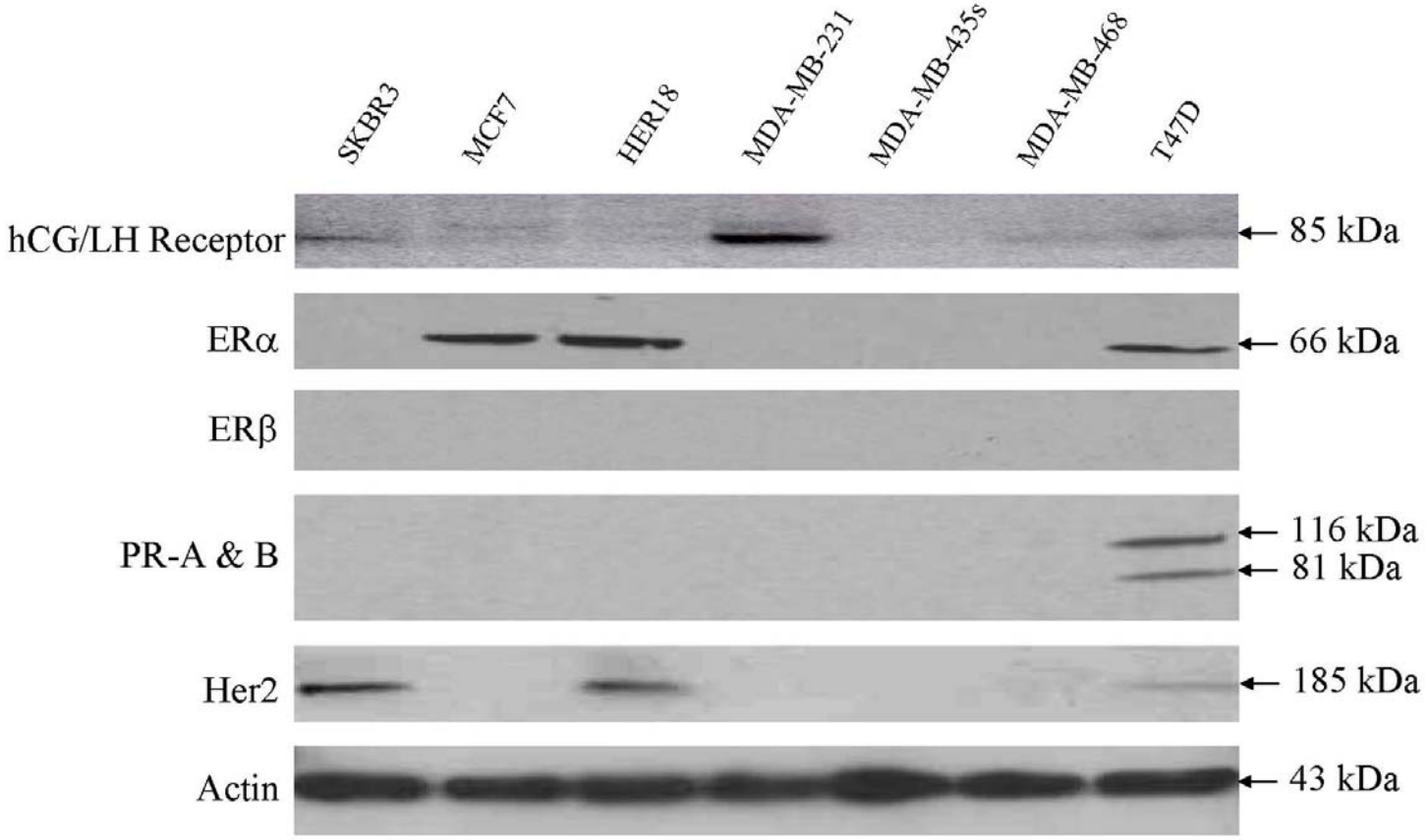

SKBR3
MCF7
HER18
MDA-MB-231
MDA-MB-435s
MDA-MB-468
T47D
hCG/LH Receptor
85 kDa
ERα
66 kDa
ERβ
PR-A & B
116 kDa
81 kDa
Her2
185 kDa
Actin
43 kDa

FIGURE LEGENDS

Fig. 1. Treatment with hCG induces apoptosis in SKBR3 breast cancer xenografts. Tumor xenografts were grown in 8-week-old athymic female nude mice as described under Materials and methods (see appended manuscript). When tumors reached 150 mm^3, 50 μL of either 100 IU of hCG or saline (control) was injected directly into matching flanks of tumor xenografts. Twenty-four 24 hrs later, the tumors were removed, fixed, and analyzed by the TUNEL assay. (A) Representative formalin-fixed sections of three matched pairs of SKBR3 xenografts tested using TUNEL assay. (B) Quantitated results. Data are presented as mean ± SEM.

Fig. 2. Ki-67 immunohistochemistry of a matched pair of SKBR3 breast cancer xenografts grown in nude mouse #5. Treatment with hCG or saline was performed as described above. H&E, TUNEL, and Ki-67 staining were obtained as described under Materials and methods (see appended manuscript). Representative images for each type of staining are shown.

Fig. 3. Effects of hCG treatment on breast cancer cell viability. Culturing and hCG treatment of the indicated breast cancer cells lines were performed as described in Materials and Methods (see appended manuscript). Cell proliferation and viability were detected using the MTT assay. The results are presented as mean ± SEM of percent viability relative to the control.

Fig. 4. Expression of hCG/LH receptor, ERα, ERβ, PR, and Her2 in breast cancer cell lines. Total cellular proteins were prepared from the indicated cell lines and analyzed by Western blotting analysis as described in Materials and Methods (see appended manuscript). Membranes were probed with antibodies specific for the indicated protein markers. Actin was used as the internal control. Representative Western blots are shown.

Fig. 5. Treatment with hCG inhibits Bcl-xL expression in SKBR3 xenografts. Nine pairs of xenografts were lysed, and equivalent amounts of total cellular proteins were analyzed by Western blotting analysis as described in Materials and Methods (see appended manuscript). Membranes were probed with Bcl-xL and beta actin specific antibodies. (A) Western blots are shown for three matching pairs. (B) Quantitated results for the nine pairs of xenografts. Data are presented as mean relative levels ± SEM, where the Western blot signal for Bcl-xL was corrected against the actin signal.

Fig. 6. Effects of hCG treatment on Bcl-xL and Bax protein expression in SKBR3 cells. Total cellular proteins were prepared from SKBR3 cells treated with and without hCG and analyzed by Western blotting analysis as described in Materials and Methods (see appended manuscript). Membranes were probed with Bcl-xL, Bax, and actin specific antibodies. Representative Western blots are shown for three matching pairs.

APPENDICES

1) **Poster**

Human Chorionic Gonadotropin (hCG) induction of apoptosis in breast cancer. W. Bradford Carter, Madhavi Sekharam, Domenico Coppola, Niranjan Yanamadra. Abstract Presentation at 29th Annual San Antonio Breast Cancer Symposium, December 14-17, 2006. San Antonio, TX

2) Manuscript

Treatment with Human Chorionic Gonadotropin Induces Apoptosis in Breast Cancer. Dayami Lopez, Madhavi Sekharam, Domenico Coppola, W. Bradford Carter. Manuscript submitted for publication review, Molecular and Cellular Endocrinology, December 7, 2007.

Human Chorionic Gonadotropin (hCG) induction of apoptosis in breast cancer

W. Bradford Carter,_ Madhavi Sekharam, Domenico Coppola, NiranjanYanamandra

Department of Interdisciplinary Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL

Background: Locally advanced breast cancer remains difficult to treat, and large tumors are less likely to be resected with negative margins. The induction of apoptosis in breast cancer immediately preceding surgery may improve local control or potentiate chemotherapy response. Human Chorionic Gonadotropin (hCG) injection directly into Kaposi s sarcoma or melanoma has been shown to increase apoptosis in these tumor types. The primary objective of this preclinical study was to determine if intratumoral injection of hCG would significantly increase the apoptotic index in breast cancer xenografts. The secondary objective was to determine if hCG altered cell viability of breast cancer cells.

Methods: Using a human breast cancer xenograft model, 5 X 106 SKBr-3 human breast cancer cells were injected subcutaneously into each flank of nu/nu mice. When the tumors reached 6 mm, 50 uL (100 U/mL) of non-recombinant, naturally occurring hCG (A.P.L., Wyeth) or saline vehicle control was injected directly into the xenograft tumors. After 24 hrs, the tumors were harvested, and the xenografts tested for proliferation (Ki-67) and apoptosis (by TUNEL assay). The apoptotic index was calculated (apoptosis/proliferation) and statistical analysis performed using paired T-test. Xenografts were also tested for Bcl-xL expression by Western blot. Five human breast cancer cells lines were treated in vitro and cell viability tested by MTT assay.

Results: Of twelve pairs of SK-Br3 xenografts tested, there were no differences in proliferation by Ki-67 determination between control and treated xenografts. Apoptosis in hCG treated xenografts compared to vehicle controls, increased from 14% (range 1 - 20%) in control xenografts to 37% (range 1 - 70%) in hCG treated tumors (p = 0.001). Bcl-xL expression decreased in 89% of pairs tested, and cell viability decreased in treated cells to 68-82% of control (p < 0.01).

Conclusions: Naturally derived hCG induces apoptosis in human breast cancer xenografts after intratumoral injection, and decreases cell viability in vitro. The mechanism appears to involve a decrease in Bcl-xL expression. Induction of apoptosis may improve the ability to resect large breast cancers with negative margins or improve the efficacy of systemic therapy in locally advanced breast cancer. Additional patient studies are necessary to elucidate the potential benefit of pre-surgical apoptotic induction in large and locally advanced breast cancer.

INTRODUCTION

•While 75-95% of breast cancers respond to neoadjuvant chemotherapy, the pathologic complete response (pCR) rate is < 30%

• Mechanisms that suppress apoptosis may be important for the development of intrinsic or acquired resistance to anti-cancer drugs

• Induction of apoptosis may act synergistically with cytotoxic chemotherapy to improve pCR rate

• Purified human Chorionic Gonadotropin (hCG) has been shown to inhibit proliferation and progression of mammary carcinoma in vivo by activation of apoptotic genes

• hCG Associated Factor (HAF) induces apoptosis is Kaposi's sarcoma and melanoma after intratumoral injection

Objectives: To determine if intratumoral injection of purified hCG increases the apoptotic index in breast cancer xenografts, and to determine if purified hCG altered cell viability of breast cancer cell lines.

Figure 1. hCG induced apoptosis in SKBr-3 mice xenografts. 5X106 SKBr-3 cells were injected s.c. into the right and left flanks of 8-week -old athymic female nude mice (Harlan). When tumors reached 150 mm^3, 50 microL of 100 U of non-recombinant, purified hCG (A.P.L., Wyeth) or saline control was injected directly into the xenograft tumors. After 24 hrs, the tumors were snap frozen or fixed for Western blots and immunohistochemistry. Apoptosis was determined by TUNEL assay. Apoptosis in hCG treated xenografts compared to vehicle controls, increased from 14% (range 1 - 20%) in control xenografts to 37% (range 1 - 70%) in hCG treated tumors (p = 0.001.

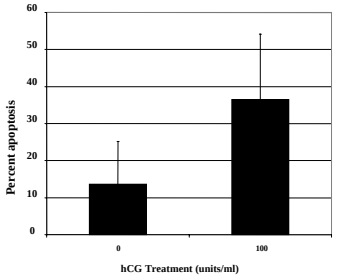


Figure 2. Immunostaining of a matched pair of SKBr-3 xenografts grown in nude mouse #5 treated with 100 U/mL hCG. Similar histologic xenograft tumors (H & E) show equal proliferation (Ki-67) but significant increase in apoptosis (TUNEL) after treatment with hCG vs saline vehicle control.

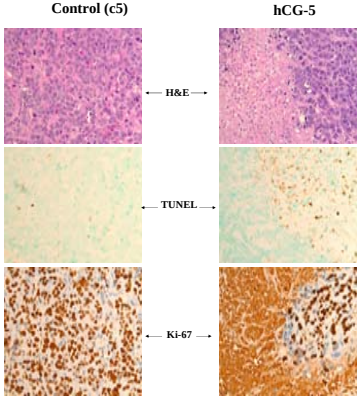


Figure 3. TUNEL assay in SKBr-3 xenografts. Formalin-fixed sections of SKBr-3 xenografts after intratumoral injection with hCG (100 U/mL) were deparaffinized and TUNEL assay was performed using the Chemicon apoptag peroxidase in situ apoptosis detection kit (TdT-mediated dUTP Nick End-Labeling). Significant increase in apoptosis is detected, depicted here by three matched pairs. Apoptosis in hCG treated xenografts compared to vehicle controls, increased from 14% (range 1 - 20%) in control xenografts to 37% (range 1 - 70%) in hCG treated tumors (p = 0.001)

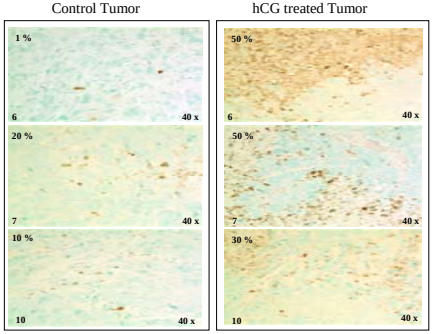


Figure 4. Cell proliferation in SKBr-3 xenografts treated with hCG. Ki-67 staining using purified anti-human Ki-67 (BD Pharmingen) at 1:400 for 30 minutes to measure proliferation in SKBr-3 xenografts was performed after intratumoral injection of purified hCG (100 U/mL). There was no difference in Ki-67 staining between hCG treated and control xenografts.

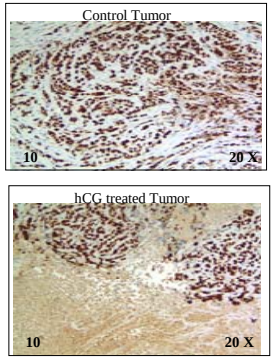


Figure 5. hCG inhibition of Bcl-XL expression in SKBr-3 xenografts. Nine pairs of xenografts were lysed, and equivalent amounts of total cellular proteins were separated by SDS-10% PAGE and transferred onto nitrocellulose membranes. The membranes were probed with primary antibodies against Bcl-xL and beta actin and detected by enhanced chemiluminescence. Bcl-xL expression decreased in 6 of 9 matched pairs tested. (H = hCG treated; C = saline treated)

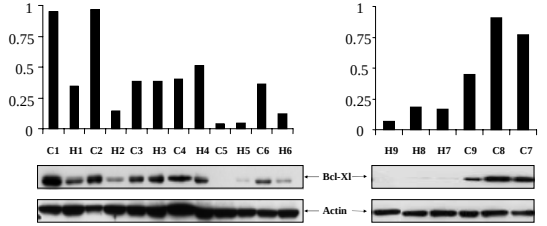
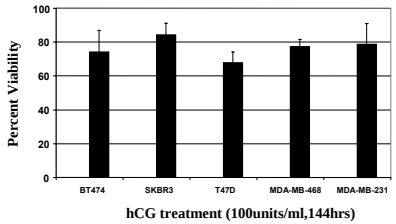


Figure 6. Breast cancer cell viability after treatment with hCG. Cell proliferation and viability were detected by Tetrazolium-based calorimetric assay (MTT). 1 x 10^4 breast cancer cells/well were seeded onto 96-well plates. After 12 hours of adherence, cells were treated with 100 U/mL of purified hCG for 6 days or vehicle, then exposed to the MTT dye (5 mg/mL) and incubated at 37°C for 3 hours. The resulting formazan crystals were solubilized with DMSO and the absorbance of each well was measured at 540 nm using a multiscan autoreader (Dynatech MR 5000, Chantilly, VA). Cell viability decreased in treated cells to 68-82% of control (p < 0.01).



CONCLUSIONS

- Intratumoral injection of purified hCG into human breast cancer xenografts increased the apoptotic index without increasing proliferation.
- Decreased expression of Bcl-xL in 67% of treated tumors suggests that this pathway may be involved in the mechanism of purified hCG induced apoptosis.
- In vitro cell viability of human breast cancer cell lines decreased significantly upon exposure to purified hCG.
- Purified hCG may be beneficial as a pro-apoptotic biological intervention. Induction of apoptosis may potentially improve the complete response rate of breast cancer during neo-adjuvant chemotherapy.
- Additional studies to evaluate the apoptotic mechanism induced by purified hCG and a possible synergy with chemotherapeutic agents is warranted.

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Manuscript Draft

Manuscript Number:

Title: TREATMENT WITH HUMAN CHORIONIC GONADOTROPIN INDUCES APOPTOSIS IN BREAST CANCER

Article Type: Research Paper

Section/Category:

Keywords: Breast cancer, hCG, receptor, Bcl-2, Bax, Bcl-XL, apoptosis, xenografts, gene expression, cell viability

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Manuscript Region of Origin:

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December 7, 2007

Dear Editor,

Attached is a copy of the manuscript entitled "**Treatment with human chorionic gonadotropin induces apoptosis in breast cancer**" by Dayami Lopez, Madhavi Sekhram, Domenico Coppola, and W. Bradford Carter for publication in Molecular and Cellular Endocrinology.

Sincerely,

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TREATMENT WITH HUMAN CHORIONIC GONADOTROPIN INDUCES APOPTOSIS IN BREAST CANCER

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Abstract

Agents that induce apoptosis in breast cancer cells have great potential to facilitate chemotherapeutic intervention and improve patient outcomes. In this study, the effects of injecting purified human chorionic gonadotropin (hCG) directly into human breast cancer xenografts grown in nude mice was examined. It was demonstrated that intra-tumoral injection of purified hCG increased the apoptotic index in breast cancer xenografts. These results were supported by the findings that exposure of breast cancer cells to purified hCG decreased cell viability in five different breast cancer cell lines. Further investigation revealed that the expression of Bcl-xL, Bcl-2, and Bax was altered in concert with their role in apoptosis as demonstrated by Western blotting analysis and immunohistochemistry. Preoperative apoptotic induction by factors such as hCG may improve local control, or work synergistically with neoadjuvant chemotherapy to improve complete pathologic response of locally advanced breast cancer.

Keywords: Breast cancer, hCG, receptor, Bcl-2, Bax, Bcl-XL, apoptosis, xenografts, gene expression, cell viability

1. Introduction

Breast cancer is the most common cancer among women and the second leading cause of cancer deaths in women after lung cancer. The American Cancer Society estimates that more than 200,000 women are diagnosed with breast cancer each year in the United States alone. The outcomes of breast cancer treatment depend greatly on response to therapy and are predicted based on lymph node status and the extent of disease. Locally advanced breast cancers remain difficult to treat, and large tumors are less likely to be resected with negative margins. Although 75-95% of locally advanced breast cancers respond to neoadjuvant chemotherapy, the pathologic complete response (pCR) rate is less than 20%. Since patients with higher pCR rate have improved outcomes, identifying mechanisms to improve the pCR rate may impact on survival of locally advanced breast cancers. Mechanisms that suppress apoptosis are suspected to be significant contributors to the development of intrinsic or acquired resistance to anti-cancer drugs and may prevent complete responses to neoadjuvant chemotherapy. Thus, the induction of apoptosis in breast cancer cells may facilitate therapeutic intervention and potentially improve outcomes.

A hormone that could be useful in the induction of apoptosis in breast cancer cells is human chorionic gonadotropin (hCG). It is well known that hCG belongs to both the glycoprotein hormone and the cysteine knot growth factor families (Lapthorn *et al.*, 1994; Pierce and Parsons, 1981). The biologically active molecule of hCG consists of two non-covalently linked subunits, the free hCG-α and hCG-β, which are encoded by several independent genes (Lapthorn *et al.*, 1994; Pierce and Parsons, 1981). The levels of hCG increase exponentially during the first trimester of pregnancy followed by a rapid decline to low steady-state levels after that (Lapthorn *et al.*, 1994; Pierce and Parsons, 1981). In addition to its pregnancy-maintaining actions, hCG

causes differentiation of the breast glandular epithelium, which in turn results in 1) inhibition of cell proliferation, 2) increase in DNA repair capabilities of this tissue, and 3) decrease in the binding of carcinogen to the mammary cell DNA (Russo and Russo, 1995; Srivastava *et al.*, 1999). Most hCG actions are mediated by a G-protein-coupled receptor, which also binds luteinizing hormone (LH) (Loosfelt *et al.*, 1989; McFarland *et al.*, 1989). In fact, consistent with hCG function in breast tissue, hCG/LH receptors have been detected in normal breast epithelial cells, breast cancer tissues, and breast cancer cell lines (Hu *et al.*, 1999; Lojun *et al.*, 1997; Meduri *et al.*, 1997, 2003; Span *et al.*, 2003; Taback *et al.*, 2001). This finding is critical considering that it has been proposed that hCG may be useful in the prevention and/or treatment of breast carcinoma (Janssens *et al.*, 2007; Meduri *et al.*, 2003; Rao, 2000; Russo and Russo, 2000). Interestingly, several studies have reported that full-term pregnancy at a young age has a perceived protective effect against the development of breast cancer (Kelsey *et al.*, 1993; Lambe *et al.*, 1996; MacMahon *et al.*, 1970; Medina, 2004; Trapido, 1983).

The aim of this study was to determine whether intratumoral injection of purified hCG could increase the apoptotic index in breast cancer xenografts, and to examine whether hCG alters the viability of different breast cancer cell lines.

2. Materials and methods

2.1 Animals

Eight-week-old athymic female nude mice were purchased from Harlan Industries (Madison, WI). All experiments involving animals were carried out according to the regulations of the University of South Florida Institutional Animal Care and Use Committee. Mice were fed Tekland 22/5 rodent chow *ad libitum* and housed in a light-controlled room with 12 hrs of light followed by 12 h of darkness and had free access to water. To make the tumor xenografts, $5X10^6$ SKBR3 cells were harvested, resuspended in PBS, and injected subcutaneously into the right and left flanks of the mice. When tumors reached 150 mm^3, 50 μL of 100 U of non-recombinant, semi-purified hCG (A.P.L., Wyeth) was injected directly into the tumor xenografts of the left flanks, whereas the tumors in the right flanks received 50 μL of saline vehicle (control). After 24 hrs, tumors were harvested, snap frozen or fixed in 10% buffered formalin, and used in the different experiments.

2.2 Materials

The human breast cancer SKBR3, MCF7, MDA-MB-231, MDA-MB-435s, MDA-MB-468, and T47D cell lines were obtained from the American Type Culture Collection (Rockville, MD). The HER18 cell line, is a MCF7 cell line that overexpress HER2 6-fold, was kindly provided by MD Anderson Cancer Center (Houston, TX). Dubecco's Modified Eagle Medium (D-MEM) and 100X antibiotic-antimycotic were from Gibco/BRL (Grand Island, NY). Fetal bovine serum, nitrocellulose membrane, and X-ray films were obtained from Fisher Scientific (Norcross, GA). The ApoTag *In Situ* Apoptosis Detection Kit and the Chemicon Mouse to

Mouse Detection Kit were obtained from Chemicon International Inc. (Temecula, CA). Methyl Green counterstain was from Vector Laboratories (Burlingame, CA). The human Ki-67 specific antibody was obtained from BD Pharmingen (San Jose, CA). The human LH receptor, progesterone receptor (PR), Her2 (Neu), Bcl-XL, Bax and β-actin specific antibodies, and the horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The human estrogen receptor α and β (ERα and ERβ, respectively) specific antibodies and the Vybrant MTT Cell Proliferation Assay kit were obtained from Invitrogen Corp (Carlsbad, CA). DAB was from DakoCytomation (Carpinteria, CA). Pre-stained protein molecular weight markers were purchased from BioRad Labs (Hercules, CA). The BCA protein assay kit, pre-cast 4-20% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), RIPA buffer, and the SuperSignal West Pico Chemiluminescent Substrate were purchased from Pierce (Rockford, IL). All other chemicals were purchased from Fisher Scientific or Sigma-Aldrich Co (St. Louis, MO).

2.3 Terminal Transferase dUTP Nick End Labeling (TUNEL) Assay

DNA fragmentation in apoptotic cells was determined by the TUNEL assay using the ApoTag *In Situ* Apoptosis Detection Kit. For this, formalin-fixed paraffin embedded sections were cut at 4-5 microns and air dried overnight at room temperature. Sections were then deparaffinized, rehydrated, and digested with proteinase K (25 ug/ml in TBS) using standard methods. After quenching with 3% hydrogen peroxide, sections were treated with equilibration buffer for 10 minutes. Incubation with TdT in the presence of modified nucleotides was carried out for 60 minutes at 37°C, which resulted in the labeling of DNA fragments with the digoxigenin-nucleotide. The reaction was stopped by incubating with stop/wash buffer for 10

minutes. On the Dako autostainer, the digoxigenin-nucleotide was incubated with anti-digoxigenin-peroxidase (diluted 1:500) for 30 minutes, and then with DAB chromogenic substrate for 10 minutes. Sections were manually counterstained with Methyl Green, dehydrated through graded alcohols, cleared in xylene, and mounted with resinous mounting medium. All slides were graded by percent of stained cells.

2.4 H&E Staining

Formalin-fixed paraffin embedded sections of tumor xenografts were cut at 3 microns and allowed to air dry. Deparaffinized, rehydrated sections were stained for 6 minutes with Mayer's Hematoxylin (modified AFIP recipe), washed in running tap water, and counterstained with Eosin-Phloxine (AFIP recipe). Sections were then dehydrated, cleared with xylene, and mounted with resinous mounting medium.

2.5 Ki-67 Immunohistochemistry

Formalin-fixed paraffin embedded sections cut at 3 microns were deparaffinized and rehydrated as described above. Sections received microwave antigen retrieval with 0.01M citrate buffer, pH 6.0 (high to boiling and then 20 minutes on power level 5, Emerson 1100W microwave). After cooling for 20 minutes, sections were rinsed with deionized water and placed in TBS/Tween for 5 minutes. Slides were immunostained on a Dako autostainer using the Chemicon Mouse to Mouse detection kit. Endogenous peroxidase was blocked with 3% aqueous hydrogen peroxide. Slides were incubated with anti-human Ki-67 (dilution 1:400) for 30 minutes. DAB was used as the chromogen. After removal from the autostainer, slides were

counterstained with modified Mayer's hematoxylin, dehydrated, cleared with xylene, and mounted with resinous mounting medium.

2.6 Tetrazolium-Based Colorimetric Assay (MTT)

All human breast cancer cells were maintained at a density of 10^8 cells per 75 cm^3 flask in high glucose D-MEM medium supplemented with 10% fetal bovine serum and 1X antibiotic-antimycotic before starting the experiments. Cell proliferation and viability were determined using the Vybrant MTT Cell Proliferation Assay kit. For this, 1 x 10^4 breast cancer cells/well were seeded onto 96-well plates in 100 µl of culture medium. After 12 hours of adherence, cells were treated with 100 units/ml of purified hCG or vehicle for 6 days. After the incubation time, cells were exposed to the MTT dye (5 mg/mL) and incubated at 37°C for 3 hours. The resulting formazan crystals were solubilized and the absorbance measured at 540 nm using a multiscan autoreader (Dynatech MR 5000, Chantilly, VA).

2.7 Western blotting analysis

Cell lysates were prepared with ice-cold RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate, 0.1% SDS and protease and phosphatase inhibitors) and clarified by centrifugation. Protein concentrations of supernatants were determined by BCA protein assay kit (Pierce). Equivalent amounts of total cellular proteins were denatured at 100°C in loading buffer and subjected to electrophoresis on a pre-cast 4-20% SDS-PAGE. After electrophoresis, samples were electroblotted onto nitrocellulose membranes (0.2 µm pore) in buffer containing 25 mM Tris hydrochloride (Tris-HCl), pH 8.3, 0.192 M glycine, and 20% methanol for 1 hr at 4°C. To verify equal protein loading, nitrocellulose membranes were

stained with 0.1% Ponceau S (in 5% acetic acid) and destained in water. Western blot analysis for the different proteins was carried out with 1:100 – 1:1000 dilutions (depending on the antibody) of antibody specific for each protein in 5% milk/TTBS. Immunoreactive proteins were visualized using a 1:10,000 dilution of horseradish peroxidase–conjugated secondary antibody in 5% milk/TTBS and the SuperSignal West Pico Chemiluminescent Substrate (Pierce). Multiple exposures ranging from 5 seconds to 20 min were made.

2.8 Statistical Analysis

Data from the individual parameters were compared by analysis of variance (ANOVA) followed by Student-Newman-Keuls multiple comparison test when applicable. A $p<0.05$ was considered significant for all tests.

3. Results

To determine whether hCG could induce apoptosis in breast cancer xenografts, 50 μL of either 100 IU of semi-purified hCG or saline control was injected into matching flanks of SKBR3 breast cancer xenografts grown in nude mice. After 24 hrs, the tumors were harvested and analyzed by TUNEL assay as described under Materials and methods. It is important to mention that there was evidence of necrosis at the injection site, but clear apoptosis was detected in viable tissue by the TUNEL assay. **Figure 1A** illustrates the results for three representative matched pairs tested using TUNEL assay. As shown, hCG-treated tumors showed 37% apoptosis (range 1-70%) within the xenografts as compared to 14% apoptosis (range 1-20%) observed in saline (control) treated tumor xenographs ($p = 0.001$, **Figure 1B**).

To examine whether the increase in apoptosis seen in **Figure 1** could be matched by an increase in cell proliferation, hCG treated and control xenografts were tested by immunocytochemistry using a Ki-67 specific antibody. Representative images of H&E, TUNEL, and Ki-67 staining are depicted in **Figure 2**. As shown, no differences were observed in cell proliferation as indicated by the Ki-67 staining patterns of saline (control) and hCG treated xenografts. However, a significant increase in TUNEL staining was observed in hCG treated xenografts when compared to control xenografts. Similar results were obtained in the other matched pairs (data not shown). These results suggest that direct, intra-tumoral injection of hCG can induce apoptosis in breast cancer cells without affecting cell proliferation.

To confirm the results obtained with the xenografts, cell viability and proliferation experiments were carried out *in vitro* using different breast cancer cell lines. SKBR3, MCF7, HER18, MDA-MB-231, MDA-MB-435s, MDA-MB-468, and T47D breast cancer cells were cultured in 96 well plates at a density of 1×10^4 cells/well and treated with hCG or vehicle

control for 6 days as described above. After the incubation period, cells were exposed to MTT dye (5 mg/ml) and incubated at 37^{o}C for 3 hrs. The resulting formazon crystals were solubilized and their absorbance measured at 540 nm as described under Materials and methods. As shown in **Figure 3**, viability decreased in all the cell lines tested. Significant decreases were observed in SKBR3 (22%, $p<0.001$), MDA-MB-231 (18%, $p<0.001$), MDA-MB-435s (14%, $p<0.01$), and T47D (32%, $p<0.001$) (**Figure 3**). This demonstrates that hCG can decrease breast cancer cell viability, and supports the findings of apoptosis seen in the xenograft experiments.

Studies were then carried out to determine whether the differences in hCG response observed in **Figure 3** were associated with hCG/LH receptor expression in these cell lines. As shown in **Figure 4**, hCG/LH receptor expression was detected in MDA-MB-231 > SKBR3 > T47D, MDA-MB-468 > MCF7 > HER18. The hCG/LH receptor levels found in the MDA-MB-231 and SKBR3 cells corresponded with the significant response of these cell lines to hCG treatment. Interestingly, T47D showed the highest response in the MTT studies but had about 50% the amount of hCG/LH receptors found in the SKBR3 cells. Another inconsistency was detected in the case of the MDA-MB-435s cells. These cells demonstrated significant response to hCG treatment in the MTT studies even with undetectable levels of the hCG/LH receptor. Even though MDA-MB-468, MCF7 and HER18 had detectable levels of hCG/LH receptor, their response in the MTT studies was relatively modest. Western blotting analysis was also carried out to confirm the markers expressed in each breast cancer cell line. As shown in **Figure 4**, human epidermal growth factor receptor 2 (Her2) was expressed in SKBR3, HER18, and T47D, whereas estrogen receptor α (ERα) was expressed in MCF7, HER18 and T47D. T47D also expressed both isoforms of the progesterone receptor (PR) (**Figure 4**). None of these protein

markers were detected in MDA-MB-231, MDA-MB-435s, and MDA-MB-468 (**Figure 4**). As expected, no ERβ was detected in any of the cell lines under analysis (**Figure 4**).

To start identifying potential mechanisms involved in the hCG-dependent induction of apoptosis in breast cancer cells, control and treated xenografts were tested for expression of the anti-apoptotic protein Bcl-xL using Western blotting analysis. Only nine pairs of tumor xenografts had sufficient tissue to prepare protein samples. **Figure 5A** depicts the results for three matched pairs of xenografts. hCG treatment decreased Bcl-xL protein levels in 6 of the 9 pairs (67%) of xenografts tested. In the 9 pairs of xenografts, as shown in **Figure 5B**, hCG treatment decreased Bcl-xL protein levels by 50%. Immunohistochemistry was performed on paraffin sections as previously described (Rochaix *et al.*, 1999). Immunostaining using a Bcl-2 specific antibody demonstrated that this protein was reduced by 53% in the viable tissue around the treatment area, whereas the pro-apoptotic protein Bax was significantly induced 1.5-fold (P=0.001) in the treatment area (data not shown). Reduction in Bcl-XL and induction in Bax protein expression in response to hCG treatment was observed *in vitro* in SKBR3 cells (see **Figure 6**). These results clearly implicate the Bcl-2 family of proteins as a potential pathway for apoptotic induction by hCG.

4. Discussion

In this report, we have demonstrated that direct injection of hCG into breast carcinoma xenografts induces apoptosis, and that exposure of breast cancer cells to purified hCG decreases cell viability in different breast cancer cell lines. These data are in correlation with previous *in vivo* experiments indicating that hCG can inhibit the progression of 7,12-dimethylbenz anthracene (DMBA) induced mammary carcinomas in rats through induction of apoptosis (Srivastava *et al.*, 1997). Furthermore, hCG treatment induced an acceleration in the expression of apoptotic genes such as TRMP2, interleukin-1 β-converting enzyme, bCl-XS, c-Myc, and P53, or an up-regulation of the synthesis of inhibin, which is known to have tumor suppressive activity (Alvarado *et al.*, 1994; Guo *et al.*, 2004; Lojun *et al.*, 1997; Russo and Russo, 1995; Srivastava *et al.*, 1997, 1998a, 1999). In agreement with our cell viability studies is the report indicating that the culturing of MCF7 cells with hCG results in a hCG/LH receptor-dependent decrease in cell proliferation and invasion across Matrigel membranes (Rao *et al.*, 2004). This finding is interesting considering that it has been shown that women with hCG/LH receptor-positive tumors have longer metastasis-free survival (Meduri *et al.*, 2003). Further confirmation of the effects of hCG in breast cancer was presented in pilot clinical studies demonstrating that hCG significantly reduced the proliferative index and the expression of both ER and PR in breast cancers independently of whether they were newly diagnosed or metastatic (Janssens *et al.*, 2007). This effect of hCG does not appear to be unique to breast cancer cells, since the free β-subunit of hCG has been shown to inhibit the growth of bladder cancer cells (Butler *et al.*, 2003).

Another tumor that has been reported to be influenced by hCG treatment is Kaposi's sarcoma (KS), the most common tumor found in patients with acquired immune deficiency syndrome (AIDS) (Gill *et al.*, 1996, 1997; Lunardi-Iskandar *et al.*, 1995). Purified hCG has been shown to

increase apoptosis in Kaposi's sarcoma cells, both *in vitro* and *in vivo* (Gill *et al.*, 1996, 1997; Lunardi-Iskandar *et al.*, 1995). Interestingly, when highly purified or recombinant hCG and the hCG subunits were used in the studies with KS, no effect was seen (Kachra *et al.*, 1997; Lunardi-Iskandar *et al.*, 1998; Pati *et al.*, 2000; Samaniego *et al.*, 1999). Furthermore, different sources of clinical-grade hCG preparations varied in their anti-KS activity (Pati *et al.*, 2000; Russo *et al.*, 1990; Samaniego *et al.*, 1999; Srivastava *et al.*, 1998a, 1998b, 1999). Attempts to decipher this contradiction lead to the identification of a hCG Associated Factor (HAF) which appears to be responsible for the apoptotic activity of the hCG preparations (Lunardi-Iskandar *et al.*, 1998; Pati *et al.*, 2000; Samaniego *et al.*, 1999). HAF is present in several commercial preparations of hCG, with A.P.L. (Wyeth), the inducing agent in this study, having the most activity. This hCG associated factor could be a peptide, an associated protein, or even a breakdown product of hCG that could be found in the urine of pregnant women (Pati *et al.*, 2000). In fact, it is known that the β subunit of hCG is susceptible to proteolytic cleavage *in vivo* that can produce peptides of the size of the HAF (Lang *et al.*, 1997). Other factors that could be found in commercial hCG in different proportions and have been shown to be toxic to KS cells, include lysosyme, low-molecular weight contaminants, and the eosinophil derived neurotoxin ribonuclease (EDNR) (Kachra *et al.*, 1997; Lang *et al.*, 1997; Masood *et al.*, 1999; Samaniego *et al.*, 1999). Although hCG itself appears to have a direct effect in breast cancer (Rao *et al.*, 2004; Janssens *et al.*, 2007), additional studies are required to identify/purify HAF and to determine its effects in breast cancer cells, either alone or in conjunction with hCG.

Another finding of the current report is that the response to hCG of several of the breast cancer cell lines tested does not appear to correlate with the expression levels of hCG/LH receptors. One possible explanation is that the endogenous production of hCG in these cell lines

masks the effects of the exogenously added hCG. In connection with this possibility, it has been reported that breast cancer cells are able to produce hCG (Bièche *et al.*, 1998). Interestingly, hCG-α is synthesized in high concentrations, especially in ERα-positive tumors (Bièche *et al.*, 1998), which could be associated with the low response of the MCF7 and HER18 cells to hCG treatment shown herein. The finding that T47D, which also express ERα, significantly responded to hCG treatment, could be related to the presence of PR. PR has been shown to regulate the expression of hCG-β (Reimer *et al.*, 2000). One inconsistency was observed in the case of the MDA-MB-435s cells, which had a significant response to hCG treatment in the MTT studies but no hCG/LH receptor protein was detected. This suggests that hCG may be able to activate a signaling pathway that is independent of the hCG/LH receptor. Further studies are required to examine this possibility.

We also initiated mechanistic studies and determined that Bcl-xL expression was decreased in six of 9 pairs of xenografts as determined by Western blotting analysis. Furthermore, we demonstrated using immunohistochemistry that Bcl-2 was reduced by 53% in the viable tissue around the treatment area, whereas Bax was significantly induced 1.5-fold in the treatment area. Induction in Bcl-XL and reduction in Bax protein expression was also observed in SKBR3 cells treated with hCG. In correlation with our findings, enhancement of Bax protein expression has been detected in hCGβ-expressing breast cancer cells undergoing significant apoptosis (Shi *et al.*, 2006). Studies have shown that Bax acts downstream of the p53-mediated apoptotic pathway (Choudhuri *et al.*, 2002; Medina and Kittrell, 2003; Modestou *et al.*, 2001; Pati *et al.*, 2004; Sivaraman *et al.*, 2001). Interestingly, the absence of p53 function is a known risk factor for spontaneous tumorigenesis in the mammary gland, and hormonal stimulation enhances tumor risk in p53-null mammary epithelial cells (Medina and Kittrell, 2003; Pati *et al.*, 2004;

Sivaraman *et al.*, 2001). In addition to altering the expression of apoptosis related genes, hCG-induced apoptosis appears to involve disruption of N-cadherin-mediated cell-cell adhesion via β-catenin (Pon *et al.*, 2005), activation of the hCG-sensitive cyclooxygenase-2 (COX-2) and gonadotropin-mediated phosphotidyl-inositol-3 kinase pathway (Pon and Wong, 2006), and the induction of the Fas-ligand system (Kayisli *et al.*, 2003). Additional mechanistic studies to define the role of the Bcl-2 family of proteins in hCG-induced apoptosis of breast cancer cells will be needed.

In summary, we have identified a significant apoptotic induction in breast cancer xenografts after direct injection of a HAF-containing preparation of hCG. While further characterization of the inducing agents is necessary, these experiments suggest a potential therapeutic advantage by intralesional injection to induce apoptosis in locally advanced breast cancer.

Acknowledgements

The authors thank Nicole Reed for the valuable technical assistance. This research was supported in part by a grant from the Department of Defense, Breast Cancer Research Program, DAMD 17-00-1-0240.

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Figure Legends

Fig. 1. Treatment with hCG induces apoptosis in SKBR3 breast cancer xenografts. Tumor xenografts were grown in 8-week-old athymic female nude mice as described under Materials and methods. When tumors reached 150 mm^3, 50 μL of either 100 IU of hCG or saline (control) was injected directly into matching flanks of tumor xenografts. Twenty-four 24 hrs later, the tumors were removed, fixed, and analyzed by the TUNEL assay. (A) Representative formalin-fixed sections of three matched pairs of SKBR3 xenografts tested using TUNEL assay. (B) Quantitated results. Data are presented as mean ± SEM.

Fig. 2. Ki-67 immunohistochemistry of a matched pair of SKBR3 breast cancer xenografts grown in nude mouse #5. Treatment with hCG or saline was performed as described above. H&E, TUNEL, and Ki-67 staining were obtained as described under Materials and methods. Representative images for each type of staining are shown.

Fig. 3. Effects of hCG treatment on breast cancer cell viability. Culturing and hCG treatment of the indicated breast cancer cells lines were performed as described in Materials and Methods. Cell proliferation and viability were detected using the MTT assay. The results are presented as mean ± SEM of percent viability relative to the control.

Fig. 4. Expression of hCG/LH receptor, ERα, ERβ, PR, and Her2 in breast cancer cell lines. Total cellular proteins were prepared from the indicated cell lines and analyzed by Western blotting analysis as described in Materials and Methods. Membranes were probed with

antibodies specific for the indicated protein markers. Actin was used as the internal control. Representative Western blots are shown.

Fig. 5. Treatment with hCG inhibits Bcl-xL expression in SKBR3 xenografts. Nine pairs of xenografts were lysed, and equivalent amounts of total cellular proteins were analyzed by Western blotting analysis as described in Materials and Methods. Membranes were probed with Bcl-xL and beta actin specific antibodies. (A) Representative Western blots are shown for three matching pairs. (B) Quantitated results for the nine pairs of xenografts. Data are presented as mean relative levels ± SEM, where the Western blot signal for Bcl-xL was corrected against the actin signal.

Fig. 6. Effects of hCG treatment on Bcl-xL and Bax protein expression in SKBR3 cells. Total cellular proteins were prepared from SKBR3 cells treated with and without hCG and analyzed by Western blotting analysis as described in Materials and Methods. Membranes were probed with Bcl-xL, Bax, and actin specific antibodies. Representative Western blots are shown for three matching pairs.

Figure 1

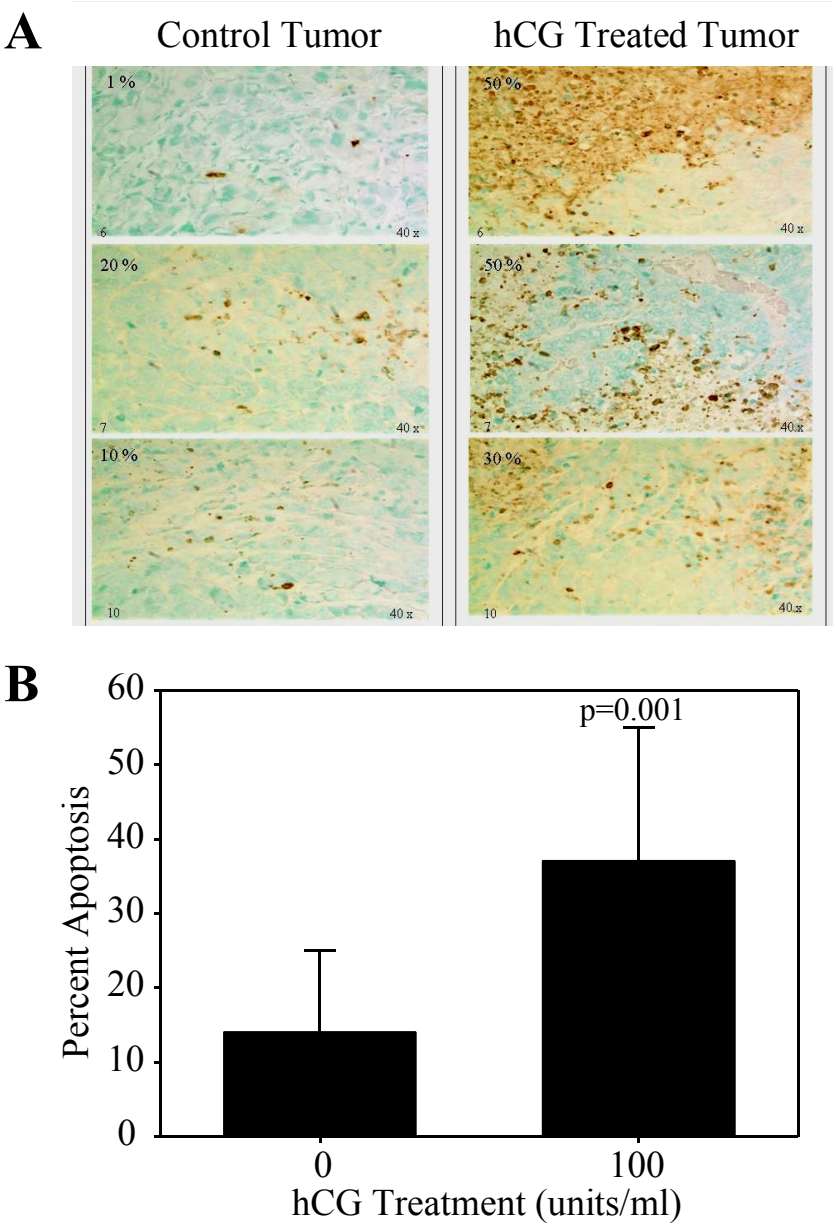

A
Control Tumor
hCG Treated Tumor
1 %
20 %
10 %
50 %
50 %
30 %
B
Percent Apoptosis
p=0.001
0
10
20
30
40
50
60
0
100
hCG Treatment (units/ml)

Figure 2

Figure 2

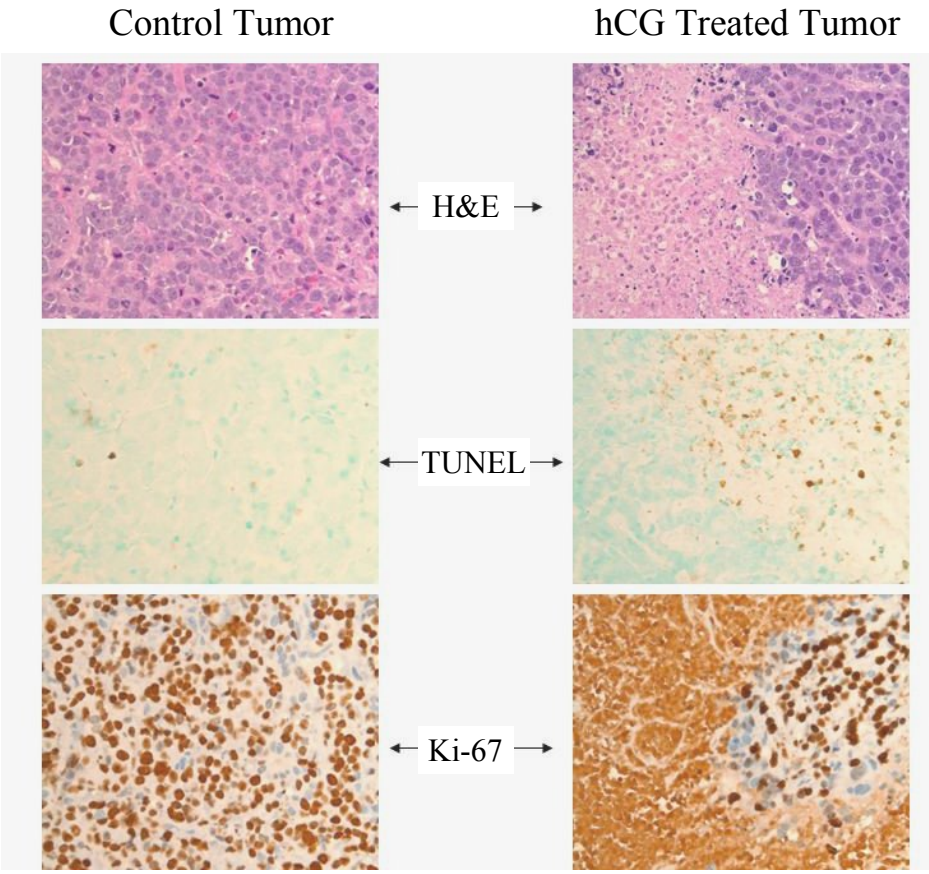


Figure 3

Figure 3

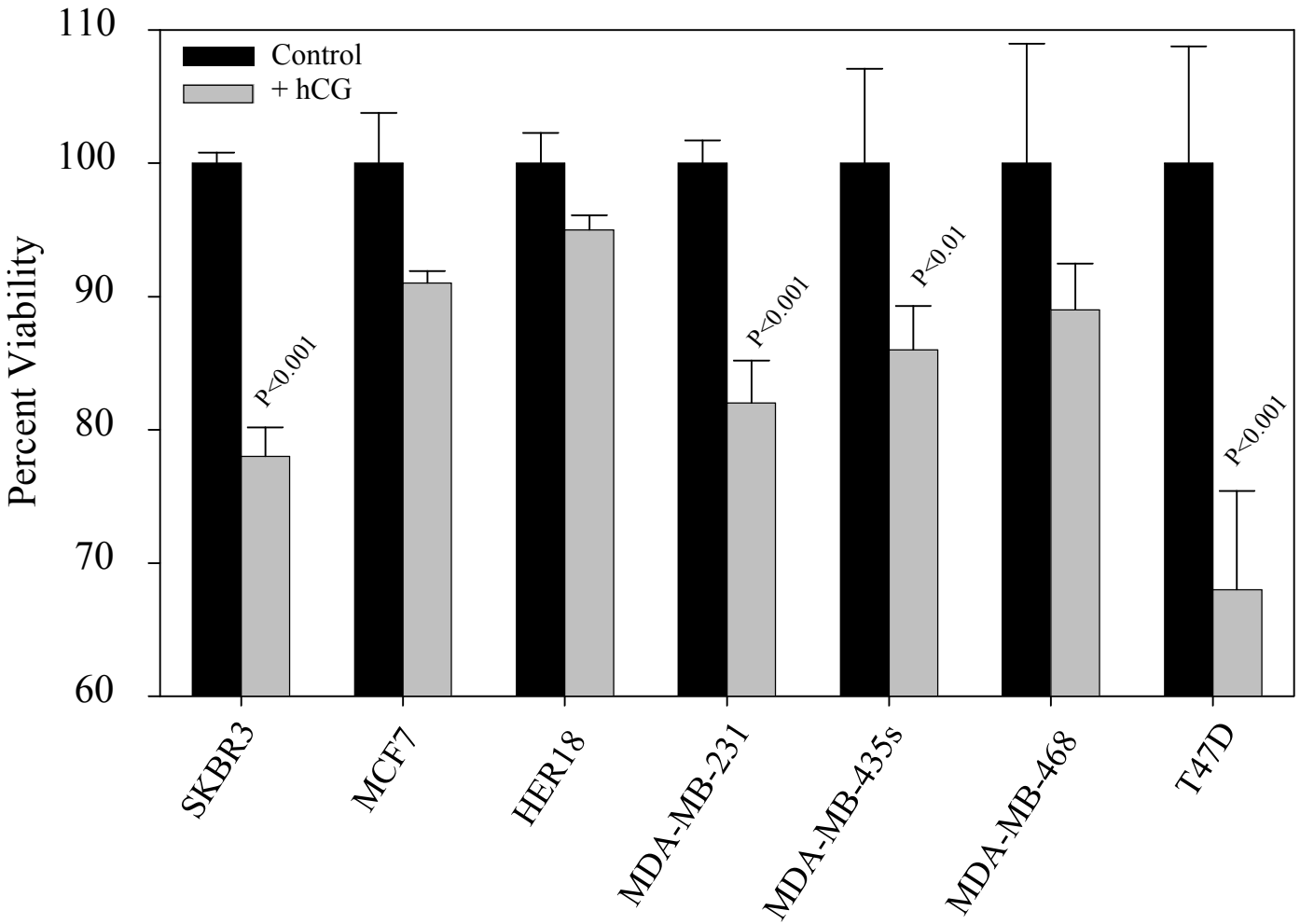


Figure 4

Figure 4

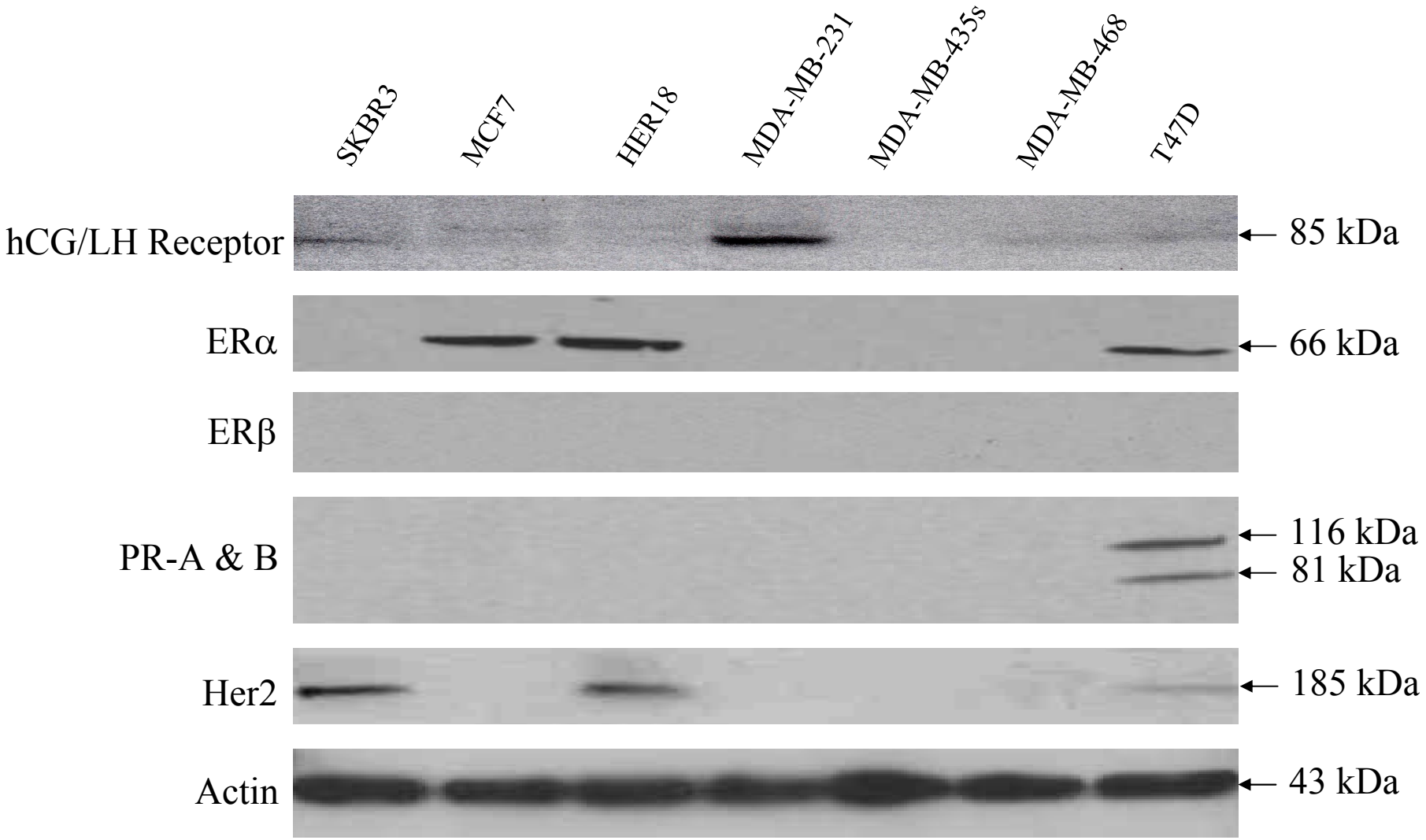


Figure 5

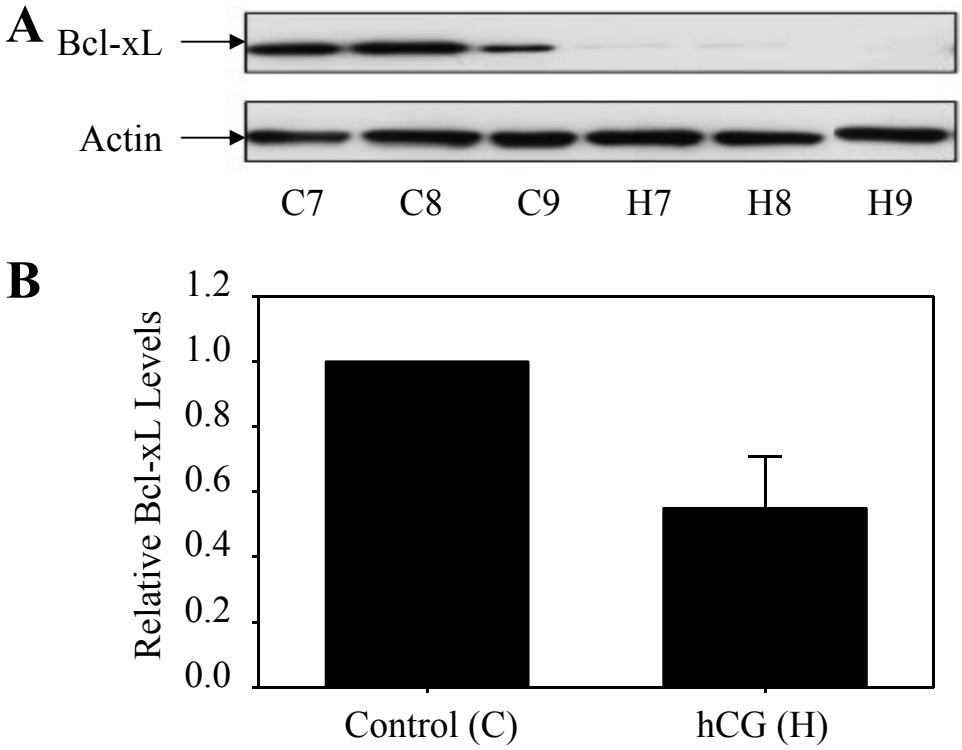
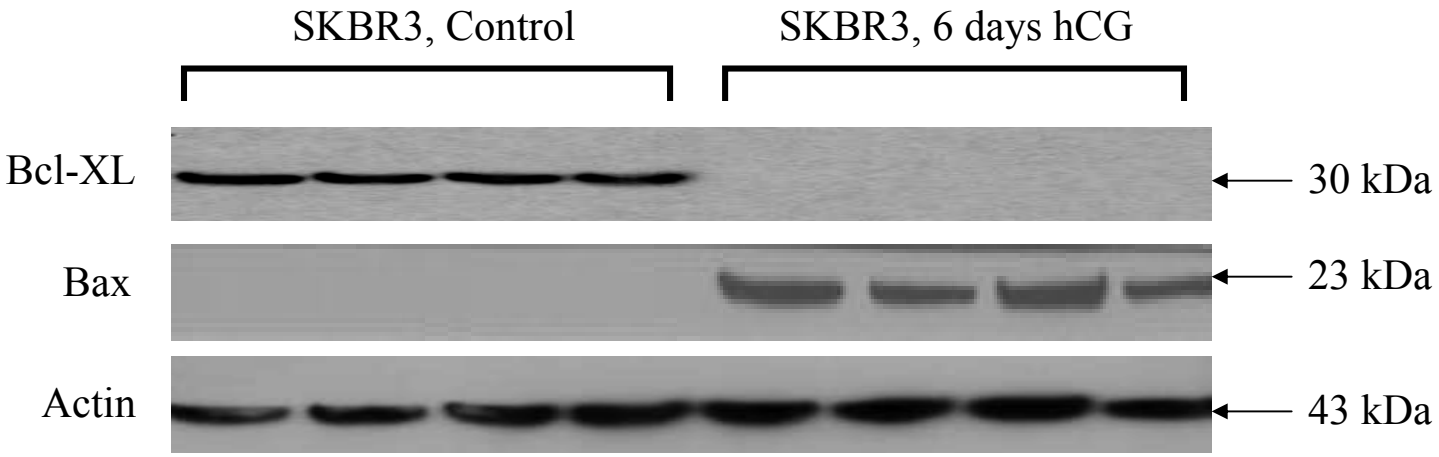

A
Bcl-xL
Actin
C7
C8
C9
H7
H8
H9
B
Relative Bcl-xL Levels
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control (C)
hCG (H)

Figure 6


SKBR3, Control
SKBR3, 6 days hCG
Bcl-XL
Bax
Actin
30 kDa
23 kDa
43 kDa